Supplementary Materials

H19 Noncoding RNA, an independent prognostic factor, regulates essential Rb-E2F and CDK8/β-catenin signaling in colorectal cancer

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Appendix A. Supplementary figures 1-7

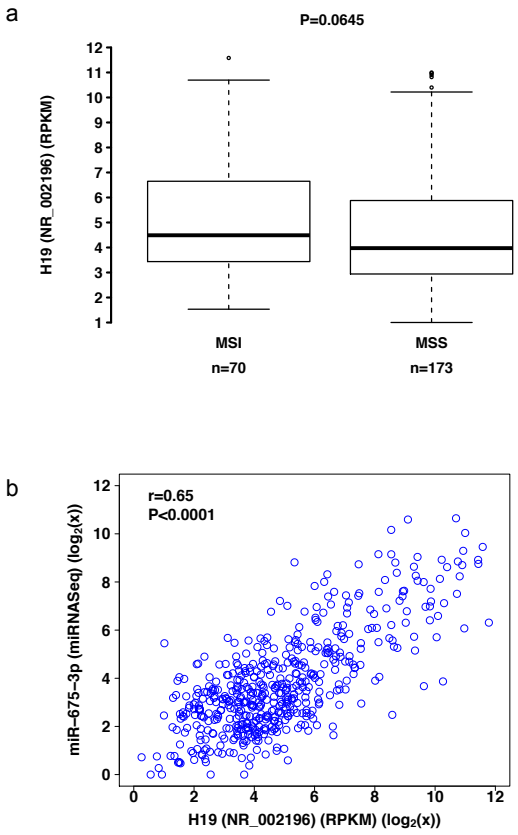


Figure S1 related to Figure 1: Association of H19 expression with microsatellite status (a), and with miR-675-3p expression (b) in TCGA CRC dataset.

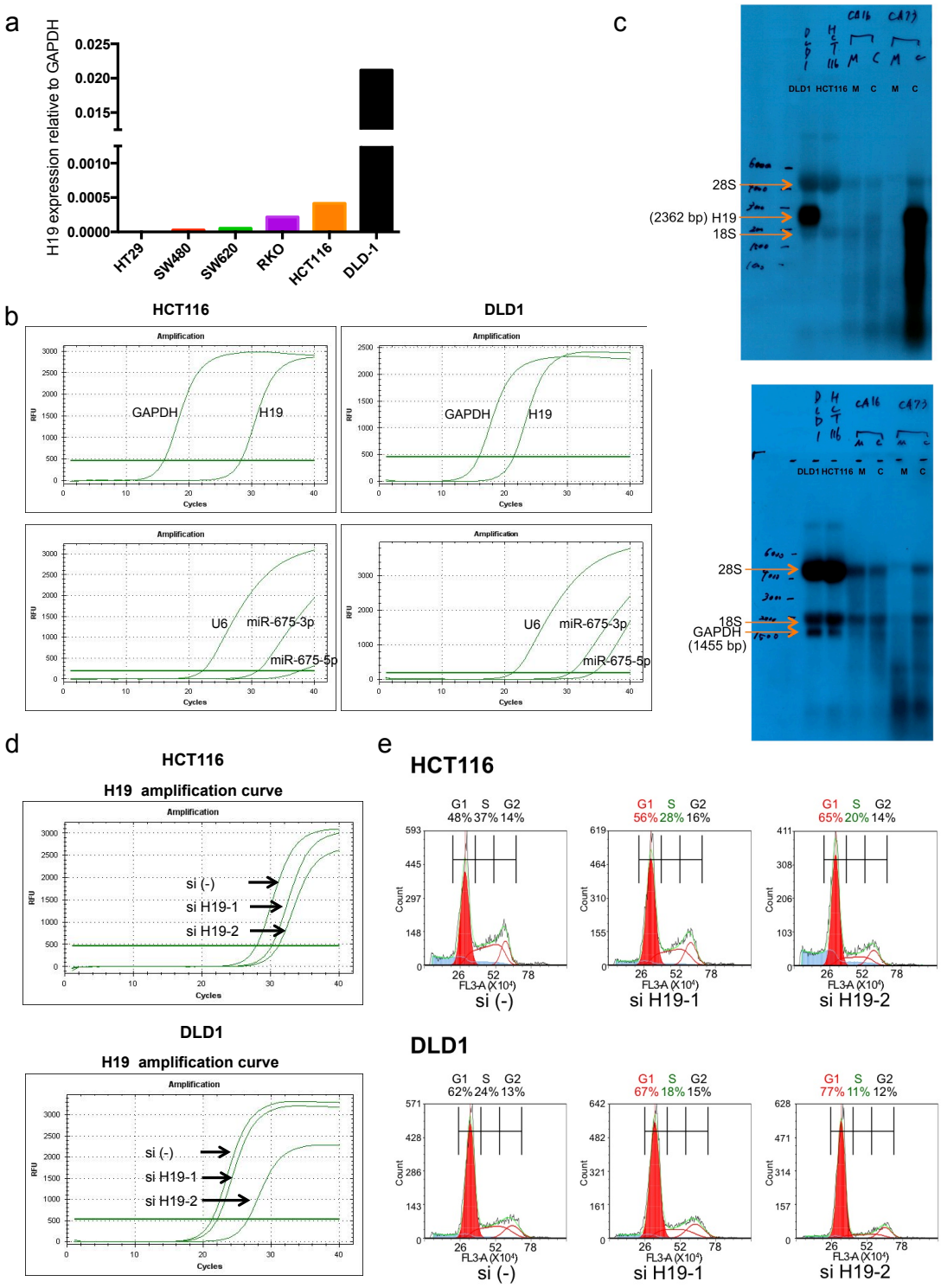
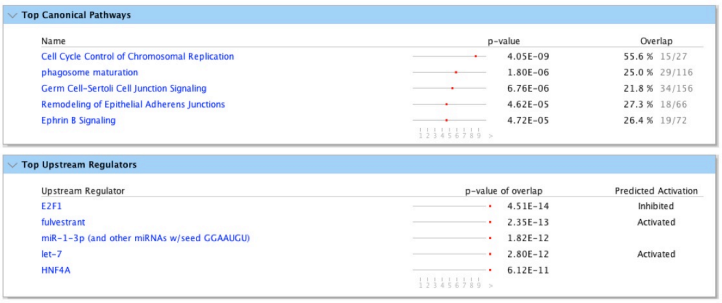


Figure S2 related to Figure 2: H19 expression in CRC cell line and effect of H19 silencing on cell cycle distribution. (a) qRT-PCR of H19 expression. (b) Amplification curve of the real-time PCR. (c) Northern blot analysis of H19 expression in CRC cell lines and paired CRC tumor (C) and mucosa (M). (d) Amplification curve of the real-time PCR. (e) H19 silencing blocked the G1-S cell cycle progression, as revealed by propidium iodide staining and flow cytometric analysis.

a

HCT116
si H19-2 vs si (-)



b

HCT116
si H19-1 vs si (-)

Upstream Regulator	Expression Fold Change	Molecule Type	Predicted function	Activation z-score	p-value of overlap
MED1	-1.200	transcription regulator	Inhibited	-2.343	1.42E-01
E2F3	1.040	transcription regulator	Inhibited	-2.573	3.96E-04
CDKN2A	1.050	transcription regulator	Activated	2.286	1.03E-02
TP53	1.140	transcription regulator	Activated	2.370	3.87E-10
E2F6	-1.180	transcription regulator	Activated	2.449	1.57E-02

c

DLD1
si H19-1 vs si (-)

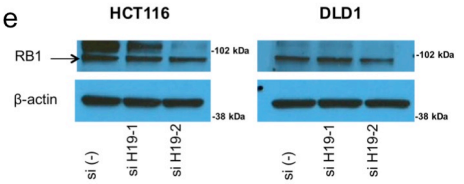
Upstream Regulator	Expression Fold Change	Molecule Type	Predicted function	Activation z-score	p-value of overlap
E2F1	-1.170	transcription regulator	Inhibited	-2.579	5.35E-05
MED1	-1.130	transcription regulator	Inhibited	-2.412	5.36E-02
E2F3	-1.020	transcription regulator	Inhibited	-2.345	1.54E-05
MYC	1.020	transcription regulator	Inhibited	-2.159	1.58E-05
E2F2	-1.150	transcription regulator	Inhibited	-2.000	3.27E-05
E2F6	-1.520	transcription regulator	Activated	2.828	7.32E-06
RBL2	-1.200	other	Activated	1.941	2.63E-01
APC	-1.100	enzyme	Activated	1.886	5.52E-03
let-7	-1.310	microrna	Activated	1.862	2.10E-04

d

DLD1
si H19-2 vs si (-)

Upstream Regulator	Expression Fold Change	Molecule Type	Predicted function	Activation z-score	p-value of overlap
E2f		group	Inhibited	-2.828	4.14E-05
E2F3	-1.040	transcription regulator	Inhibited	-2.894	1.63E-03
MYC	-2.120	transcription regulator	Inhibited	-2.663	2.86E-06
MED1	-1.110	transcription regulator	Inhibited	-2.219	4.68E-02
E2F6	-1.400	transcription regulator	Activated	2.646	9.01E-05
TP53	1.190	transcription regulator	Activated	1.592	1.36E-07

e



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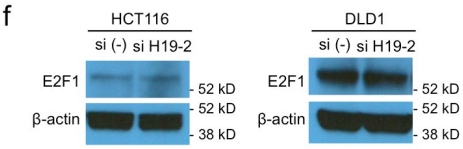

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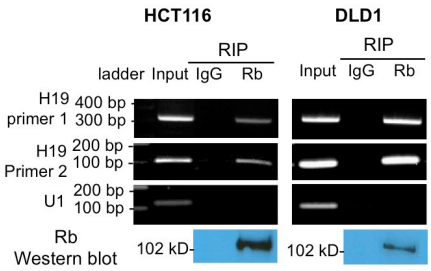
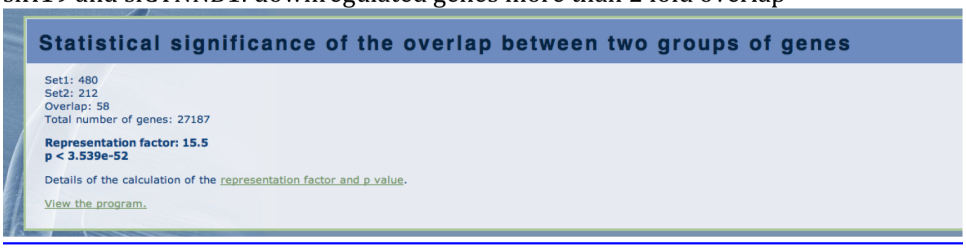


Figure S3 related to Figure 3: Effect of H19 on RB1-E2F signaling. (a) Summary page of the ingenuity pathway analysis showing predicted pathway changes, and E2F1 as most significant upstream regulator. (b) Upstream regulator prediction with HCT116 si H19-1 array data. (c) Upstream regulator prediction with DLD1 si H19-1 array data. (d) Upstream regulator prediction with DLD1 si H19-2 array data. (e, f) H19 does not change RB1 or E2F1 protein expression. The E2F activator including E2F1, E2F2, and E2F3 are predicted as inhibited, and E2F inhibitor including E2F6 and RBL2 are predicted as activated. (g) H19 interaction with RB1, determined with RNA immunoprecipitation assay. U1 as negative control.

http://nemates.org/MA/progs/overlap_stats.html

siH19 and siCTNNB1: downregulated genes more than 2 fold overlap



siH19 and siCTNNB1: UPregulated genes more than 2 fold overlap

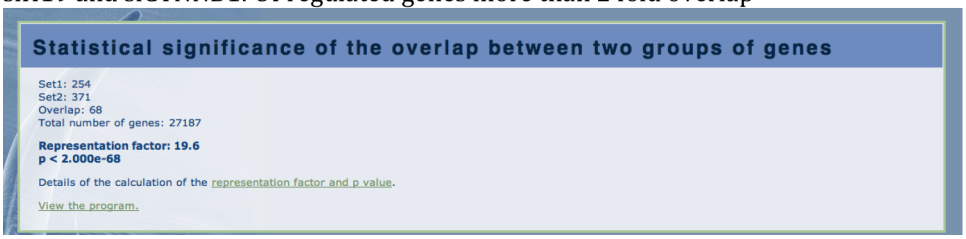
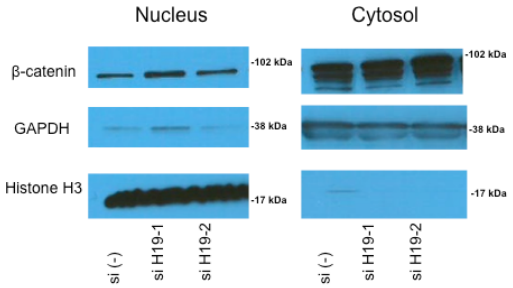


Figure S4 related to Figure 4: Statistically analysis on the overlap of regulated genes between H19 and CTNNB1. The overlap is significant ($p < 5.357e-89$; representation factor 17.8, a representation factor > 1 indicates more overlap than expected of two independent groups); and ($p < 7.483e-72$; representation factor 17.6, a representation factor > 1 indicates more overlap than expected of two independent groups) as determined using the online program of statistical significance of the overlap between groups of genes (http://nemates.org/MA/progs/overlap_stats.html).

a



b

HCT116 si H19-1 vs si (-)

Upstream Regulator	Expression Fold Change	Molecule Type	Predicted function	Activation z-score	p-value of overlap
MED1	-1.200	transcription regulator	Inhibited	-2.343	1.42E-01
E2F3	1.040	transcription regulator	Inhibited	-2.573	3.96E-04
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TP53	1.140	transcription regulator	Activated	2.370	3.87E-10
E2F6	-1.180	transcription regulator	Activated	2.449	1.57E-02

c

DLD1 si H19-1 vs si (-)

Upstream Regulator	Expression Fold Change	Molecule Type	Predicted function	Activation z-score	p-value of overlap
E2F1	-1.170	transcription regulator	Inhibited	-2.579	5.35E-05
MED1	-1.130	transcription regulator	Inhibited	-2.412	5.36E-02
E2F3	-1.020	transcription regulator	Inhibited	-2.345	1.54E-05
MYC	1.020	transcription regulator	Inhibited	-2.159	1.58E-05
E2F2	-1.150	transcription regulator	Inhibited	-2.000	3.27E-05
E2F6	-1.520	transcription regulator	Activated	2.828	7.32E-06
RBL2	-1.200	other	Activated	1.941	2.63E-01
APC	-1.100	enzyme	Activated	1.886	5.52E-03
let-7	-1.310	microrna	Activated	1.862	2.10E-04

d

DLD1 si H19-2 vs si (-)

Upstream Regulator	Expression Fold Change	Molecule Type	Predicted function	Activation z-score	p-value of overlap
E2f		group	Inhibited	-2.828	4.14E-05
E2F3	-1.040	transcription regulator	Inhibited	-2.894	1.63E-03
MYC	-2.120	transcription regulator	Inhibited	-2.663	2.86E-06
MED1	-1.110	transcription regulator	Inhibited	-2.219	4.68E-02
E2F6	-1.400	transcription regulator	Activated	2.646	9.01E-05
TP53	1.190	transcription regulator	Activated	1.592	1.36E-07

e

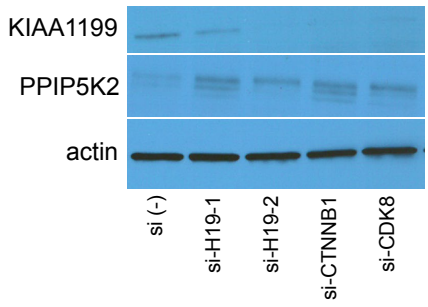

Figure S5 related to Figure 5: Effect of H19 on CDK8 and β-catenin activity. (a) Silencing H19 does not reduce nuclear expression of β-catenin protein. (b) Upstream regulator prediction with HCT116 si H19-1 array data. (c) Upstream regulator prediction with DLD1 si H19-1 array data. (d) Upstream regulator prediction with DLD1 si H19-2 array data. MED1, reflecting mediator activity of CDK8, is predicted as inhibited, and APC, the negative regulator of β-catenin, is predicted as activated. (e) The effect of H19 knockdown, CTNNB1 knockdown and CDK8 knockdown on KIAA1199 and PPIP5K2 in HCT116 cells.

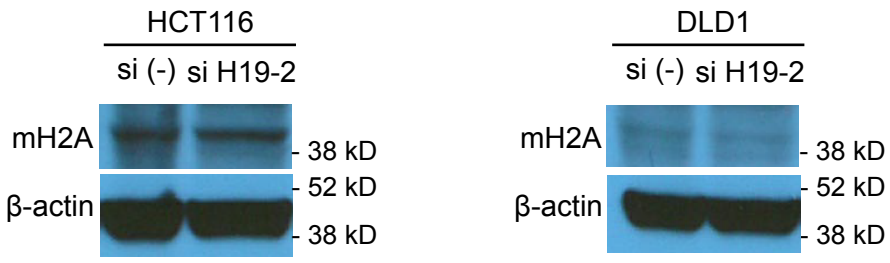

Figure S6 related to Figure 6: Silencing H19 does not significantly change macroH2A protein levels.

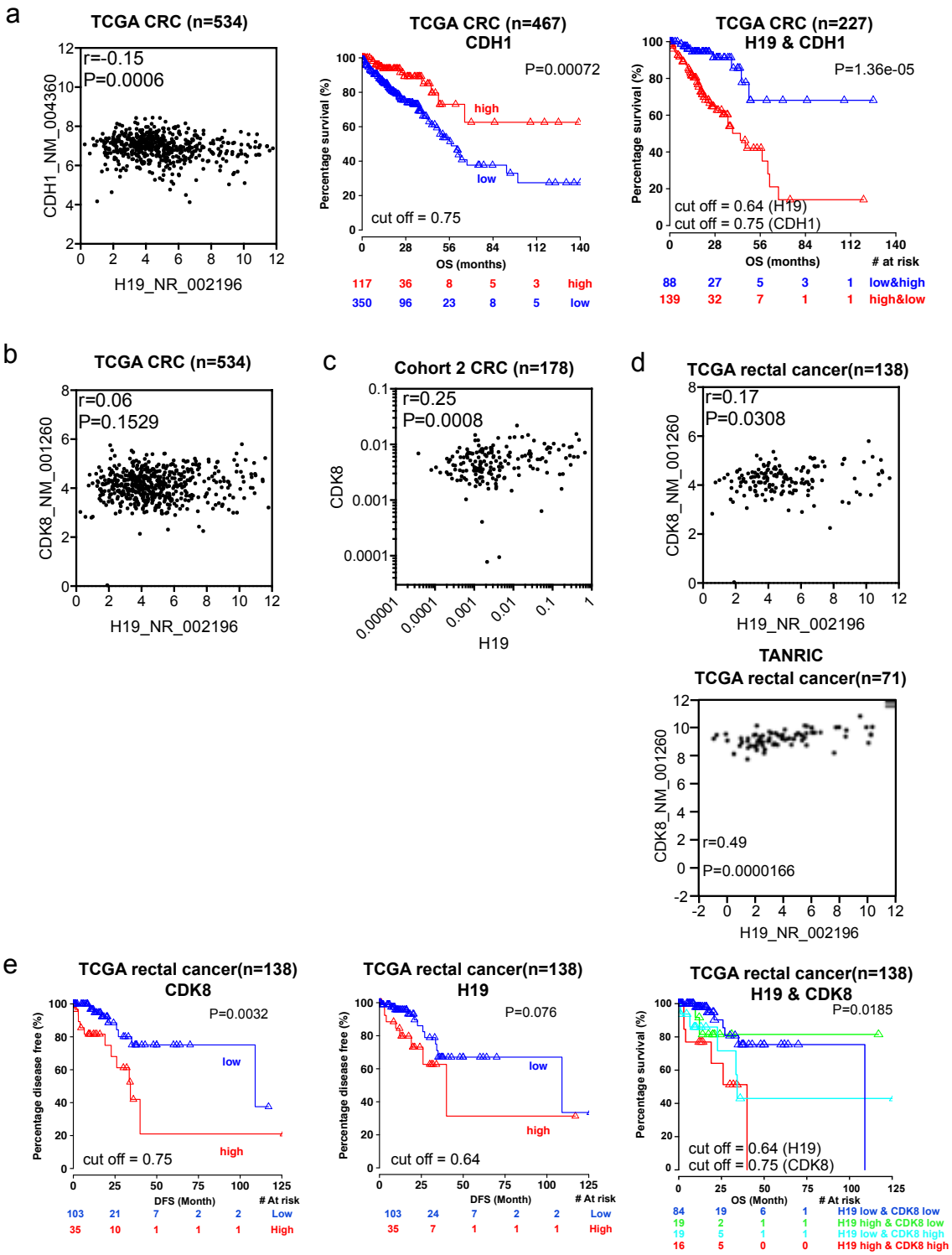

Figure S7 related to Figure 7: Clinical significance of H19 and its molecular targets in CRC. (a) Expression correlation of H19 and CDH1, and their association with overall survival in TCGA CRC dataset. (b, c) Association of H19 and CDK8 RNA expression in CRC samples. (d) Significant expression correlation between H19 and CDK8 in TCGA rectal cancer samples analyzed by us (upper image) or retrieved by TANRIC open-access resource (lower image). (e) Association of CDK8 and H19 with shorter disease-free survival in TCGA rectal cancer cases.

Appendix B. Supplementary tables 1-5

Table S1 related to Figure 1: Main characteristics of patients and tissue samples

	TCGA (n=534)	Cohort 2 (n=178)	Cohort 3 (n=117)
Median age (range) years	66.5 (31-90)	67.9 (37-92)	64.8 (18-88)
SEX			
Male	281 (52.6%)	104 (58.4%)	70 (59.8%)
Female	250 (46.8%)	74 (41.6%)	47 (40.2%)
Not known	3 (0.6%)	0	0
Tumor location			
Colon	381 (71.3%)	110 (61.8%)	117 (100%)
Rectum	153 (28.7%)	67 (37.6%)	
Not known		1 (0.6%)	
Stage			
I	88 (16.5%)	34 (19.1%)	31 (26.5%)
II	198 (37.1%)	55 (30.9%)	38 (32.5%)
III	159 (29.8%)	50 (28.1%)	18 (15.4%)
IV	76 (14.2%)	37 (20.8%)	30 (25.6%)
Not known	13 (2.4%)	2 (1.1%)	0
MSI status			
MSI-H	32 (6.0%)		
MSI-L	38 (7.1%)		
MSS	171 (32.0%)		
Not known	293 (54.9%)		

MSS, microsatellite stable; MSI-H, microsatellite instability-high; TCGA, The Cancer Genome Atlas.

Table S2 related to Figure 7: Univariate and multivariate analysis of clinical parameters and gene expression levels with overall survival of colorectal cancer patients.

	UNIVARIATE			MULTIVARIATE	
cohort	Variable	HR(95%CI)	p-value	HR(95%CI)	p-value
TCGA	Age at Diagnosis	1.03(1.005,1.057)	0.0177	1.033(1.01, 1.06)	0.01343
	Gender (Male vs Female)	1.09(0.62,1.93)	0.757		
	Tumor location (Right vs Left)	1.66(0.94,2.96)	0.0826		
	Tumor Stage(Stage III-IV vs Stage I-II)	2.72(1.52,4.87)	7.43E-04	2.66(1.445,4.9)	0.001690
	H19 & CSRP2 (high&high vs low&low)	**3.55(2.003,6.31)**	**1.48E-05**	**2.532(1.39, 4.61)**	**0.00242**
	(cut-off=0.64, cut-off=0.71)				

	UNIVARIATE			MULTIVARIATE	
cohort	Variable	HR(95%CI)	p-value	HR(95%CI)	p-value
TCGA	Age at Diagnosis	1.04(1.01,1.065)	0.004829	1.03(1.009,1.063)	0.0093
	Gender (Male vs Female)	1.1(0.63,1.89)	0.7465		
	Tumor location (Right vs Left)	1.61(0.93,2.78)	0.0875		
	Tumor Stage(Stage III-IV vs Stage I-II)	2.27(1.28,4.02)	5.17E-03	2.56(1.43,4.59)	0.001500
	H19 & CDH1 (high&low vs low&high)	**4.45(2.09,9.45)**	**0.000106256**	**3.955(1.851, 8.451)**	**0.000386**
	(cut-off=0.64, cut-off=0.75)				

Table S3 related to Experimental Procedures: Reagents used in this study.

Category	Name	Cata log number	Concentration used	Company
siRNA	si-H19-1	SASI_Hs02_00375635	50 nM	Sigma
siRNA	si-H19-2	SASI_Hs02_00375637	50 nM	Sigma
siRNA	si-CTNNB1	SASI_Hs02_00117960	50 nM	Sigma
siRNA	si-CDK8	SASI_WI_00000017	50 nM	Sigma
Chemical	Actinomycin D	A1410	10 μg/ml	Sigma
Chemical	Mytomycin C	M0503	1 μg/ml	Sigma

Table S4 related to Experimental Procedures: Primers used in this study.

Applications	Name	Forward primer	Reverse primer
qRT-PCR	H19 primer 2	TGCTGCACTTTACAACCACTG	ATGGTGTCTTTGATGTTGGGC
qRT-PCR	CDK8	ACCTGTTTGAATACGAGGGCT	TGCCGACATAGAGATCCCAGT
qRT-PCR	CTNNB1	CCCACTGGCCTCTGATAAAGG	ACGCAAAGGTGCATGATTTG
qRT-PCR	CDH1	TGAAGGTGACAGAGCCTCTGGAT	TGGGTGAATTCGGGCTTGTT
qRT-PCR	CSRP2	TGGGAGGACCGTGTACCAC	CCGTAGCCTTTTGGCCCATA
qRT-PCR	JAG1	GAAACAGCTCGCTGATTGCT	ACCAAGCAACAGATCCAAGC
qRT-PCR	IGF1R	TCGACATCCGCAACGACTATC	CCAGGGCGTAGTTGTAGAAGAG
qRT-PCR	E2F1	ACGTGACGTGTCAGGACCT	GATCGGGCCTTGTTTGCTCTT
qRT-PCR	GAPDH	ACCCAGAAGACTGTGGATGG	TCTAGACGGCAGGTCAGGTC
RIP	H19 primer 1	GGCAAGAAGCGGGTCTGTTT	TGGCCATGAAGATGGAGTCG
RIP	H19 primer 2	TGCTGCACTTTACAACCACTG	ATGGTGTCTTTGATGTTGGGC
RIP	U1	GGGAGATACCATGATCACGAAGGT	CCACAAATTATGCAGTCGAGTTTCCC
ChIP	JAG1-TSS	TGCGCAGCCTTTTATTCCCT	GAGCACGCCCTCTCATGAAT
ChIP	JAG1-TBE	CCCTTCAAAGGAAGTCGATG	AGCCTCTGCTAACCCCTCTC
ChIP	CDK8-TSS	CTGGTTCCACGTGGTGCATTT	CCACACTCTAGCCCGCTC
ChIP	CDK8-E2F1	TTCCACGTGGTGCATTTGGC	GCACAACAGCCGGTACCTC
Nothern blot	H19 primer 1	GGCAAGAAGCGGGTCTGTTT	TGGCCATGAAGATGGAGTCG
Nothern blot	GAPDH	ACCCAGAAGACTGTGGATGG	TCTAGACGGCAGGTCAGGTC

Table S5 related to Experimental Procedures: Antibodies used in this study.

Applications	Name	Catalog No.
Western blot	β-actin	Sigma A1978
Western blot	β-catenin	Santa Cruz sc-47752 or Cell Signaling #9562
Western blot	CDK4	Santa Cruz sc-260
Western blot	CDK8	Cell Signaling #4106
Western blot	CSRP2	Abcam ab178695
Western blot	Cyclin D1	Cell Signaling #2978T
Western blot	E-cadherin	Cell Signaling #3195
Western blot	E2F1	Cell Signaling #3742
Western blot	GAPDH	Santa Cruz sc-51905
Western blot	Histone H3	Cell Signaling #9717
Western blot	IGF1R	Cell Signaling #3027
Western blot	Jagged1	Cell Signaling #2620
Western blot	KIAA1199	ThermoFisher PA5-24444
Western blot	MacroH2A	Santa Cruz sc-377452 X
Western blot	Phospho-Rb (Ser608)	Cell Signaling #2181
Western blot	PPIP5K2	Bethyl lab A304-166A-T
Western blot	Rb	Cell Signaling #9309
RIP	MacroH2A	Santa Cruz sc-377452 X
RIP	Rb	Cell Signaling #9309
ChIP	β-catenin	Millipore 05-613
ChIP	Polymerase II	Millipore 05-623

Appendix C. Supplementary Experimental Procedures

Cell Culture
The human colon cancer cell lines HCT116 and DLD1 were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown as suggested by the supplier. Cells were cultured at 37^{o}C in 5% CO_2. All cell lines were validated by The Characterized Cell Line Core at MD Anderson Cancer Center using STR DNA fingerprinting.

RNA interference experiments
HCT116 and DLD1 cells were transfected with a final concentration of 50 nM siRNA with Lipofectamine RNAiMAX reagent (Invitrogen) in Opti-MEM medium (Thermo Fisher Scientific) according to the manufacture's protocol. The siRNAs specially targeting *H19*, CTNNB1, CDK8, and E2F1 were obtained from Sigma-Aldrich (**Table S3**).

Proliferation Assay. After siRNAs treatment, CRC cells were seeded at a density of 1000 cells per well in 96-well cell culture plate. The viability of the cells was assessed by cell counting kit 8 (CCK8) (Dojindo Molecular Technical, Inc.) for 6 days or 7days.

Cell Scratch Assay. After siRNAs treatment, cells were seeded and grown to 90% confluence in 6 well plates. We pretreat cells with 1 μg/ml Mytomycin C to block proliferation, and used a 200 μl pipet tip to generate gap in the cell monolayer. Images were captured as indicated time points afterwards. Cell migration area was quantified using Image J software.

Cell-Cycle Assay. After siRNAs treatment, cells were seeded into 10 cm dish and cultured at 60% confluent. The cells were washed with phosphate buffered saline (PBS), and then fixed in 70% ethanol overnight at 4°C. The fixed cells were washed, resuspended in PBS, and incubated with 20 μg/ml of RNase A and 50 μg/mL propidium iodide (PI) for 15 min in the dark. Subsequently, the processed samples were subjected by flow cytometric analysis.

Click-iT EdU assay. After siRNAs treatment, cells were pulsed for 30 minutes with 10 μM 5-ethynyl-2´-deoxyuridine (EdU) (Click-iT EdU kit, Catalogue number C10424, Life Technologies). Media was replaced and cells were washed by 3% BSA in PBS, and then fixed by 4% paraformaldehyde for 15 minutes at room temperature. Cells were treated by 0.5% TritonX-100 in PBS and then incubated at room temperature for 20 minutes. We used Click-iT AlexaFluor 488 for staining of cells with EdU incorporations following the manufacturer's protocol. and DNA content was simultaneously measured with with Hoechst 33342. The samples were analyzed by flow cytometric analysis with the equipment LSRFortessa^{TM}X-20.

Reverse Transcription Quantitative RT-PCR Analysis
Total RNA was extracted using the TRIzol protocol (Invitrogen) and DNase-digested (Ambion); the quality of the RNA was assessed using agarose gel electrophoresis. cDNA was generated from 1 µg total RNA using the SuperScript III cDNA kit (Invitrogen) according to the manufacture´s protocol. Quantitative RT-PCR was performed using SsoAdvanced^{TM} Universal SYBR$^{®}$ Green Supermix (Bio-Rad) on CFX 384 Real Time PCR Detection System (Bio-Rad) and relative gene expression levels were measured, according to the $^{2-\Delta\Delta ct}$ method. Primer sequences are available in **Table S4**.

Notch luciferase assay: After siRNA treatment, cells were seed and transfected with Cignal RBP-Jk Reporter (luc) Kit (Qiagen). 24 h after transfection, the luciferase activity of the reporter was analyzed with Dual-Glo® Luciferase Assay System (Promega), according to the manufacturer's instruction.

Northern Blot. Twenty μg of total RNA was mixed with 30 µl RNA Sample Loading Buffer (Invitrogen) and 10 µl Blue/Orange Loading Dye (Promega), denatured for 10 min at 65°C and separated on the gel (1.5g Agarose, 10×MOPS and H_2O) and transferred BrightStar®-Plus Positively Charged Nylon Membrane (Ambion) in 0.5× TBE (Tris-borate-EDTA) buffer overnight. The RNA was cross-linked to the membrane matrix under UV (120000 μ J/cm^2). The membrane was prehybridized with ULTRAhyb®-Oligo (Ambion) and then blotted with α-32P–labeled H19 specific probe using Primer-it Ⅱ Random Primer Labeling Kit (Agilent) overnight at 68 °C. The hybridization

signals were visualized by exposure to X-ray film (Kodak, USA). GAPDH was used as the internal reference. Primer sequences for generating the probes are available in **Table S4**.

Plasmid and Virus Generation. The lentivirus expression plasmids including pLOC-CDK8 (Clone ID: ccsbBroadEn_00281) and negative control vector pLOC-RFP were purchased from Dharmacon. We produced virus soup in 293 FT cells according to the instructions of the manufacturer, and used it to induce CDK8 expression in CRC cells.

Western Blot

Total proteins were extracted with Cell Lysis Buffer (Cell Signaling) containing Protease Inhibitor Cocktail (Sigma) and Phosphatase Inhibitor Cocktail 2 (Sigma). 40 μg of total proteins mixed with Pierce™ Lane Marker Reducing Sample Buffer (Thermo Scientific) were heated at 95 °C for 5 minutes, separated by a 4–20% Criterion TGX Precast Gel (Bio-rad), and transferred onto Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs (Bio-rad). The membrane was blocked with 5% non-fat milk (Bio-rad), incubated with primary antibodies and secondary antibodies, and detected using the Thermo Scientific™ SuperSignal™ West Femto Chemiluminescent Substrate (Thermo scientific). The list of antibodies can be found in **Table S5**.